INFECTION AND IMMUNITY, Mar. 2003, p. 1569–1573 Vol. 71, No. 3
0019-9567/03/$08.00+0 DOI: 10.1128/IAI.71.3.1569–1573.2003


# Interleukin-6-Deficient Mice Are Highly Susceptible to *Giardia lamblia* Infection but Exhibit Normal Intestinal Immunoglobulin A Responses against the Parasite

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Received 17 June 2002/Returned for modification 17 September 2002/Accepted 19 November 2002

**In the present study, interleukin-6 (IL-6)-deficient mice were infected with *Giardia lamblia* clone GS/M-83-H7. Murine IL-6 deficiency did not affect the synthesis of parasite-specific intestinal immunoglobulin A. However, in contrast to wild-type mice, IL-6-deficient animals were not able to control the acute phase of parasite infection. Reverse transcription-PCR-based quantitation of cytokine mRNA levels in peripheral lymph node cells exhibited a short-term up-regulation of IL-4 expression in IL-6-deficient mice that seemed to be associated with failure in controlling the parasite population. This observation suggests a further elucidation of IL-4-dependent, Th2-type regulatory processes regarding their potential to influence the course of *G. lamblia* infection in the experimental murine host.**

In the last few years, multiple studies have addressed the ability of the protozoan intestinal parasite *Giardia lamblia* to alter its surface antigen properties (4, 12, 15). These studies have revealed that antigenic variation is associated with a unique family of surface antigens named VSP (variant surface protein). Antigenic variation has been extensively studied by performing experimental infections in the mother-offspring mouse system (4, 7, 8, 21) and by using the *G. lamblia* clone GS/M-83-H7 (human isolate) as a model parasite (1). *G. lamblia* GS/M-83-H7 essentially expresses variant surface antigen VSP H7, which is defined by its immmunoreactivity to monoclonal antibody (MAb) G10/4 (1). Previous experimental *G. lamblia* GS/M-83-H7 infections in both the mother-offspring mouse system (7, 21) and the adult mouse system using animals with a deficiency in antibody production (22) revealed that antigenic diversification of the intestinal parasite population relies on the growth-selective influence of anti-VSP H7 immunoglobulin A (IgA).

In this paper, we describe a comparative study on *G. lamblia* GS/M-83-H7 infections in wild-type adult C57BL/6 mice (purchased from RCC Ltd., Füllingsdorf, Switzerland) and interleukin-6 (IL-6)-deficient transgenic 129Sv × C57BL/6 mice (10) backcrossed onto the C57BL/6 background for seven generations (kindly provided by M. Kopf, Molecular Biomedicine, Department of Environmental Sciences, Swiss Federal Institute of Technology, Zürich, Switzerland). IL-6 has been characterized as a multifunctional cytokine that is produced by a large number of cell types, including T cells and macrophages (2). This cytokine is also supposed to participate in several immunological processes, such as inflammatory responses and B-cell differentiation (2). In several prior studies, IL-6 was identified as an immunological factor involved in the Th2-type immune response in that it, e.g., stimulates the clonal expansion of IgA-producing B cells as well as the maturation of IgA-secreting plasma cells (3, 11). By the simultaneous immunization of IL-6-deficient and wild-type mice with ovalbumin, the cytokine was also shown to be causatively linked to enhanced mucosal IgA production (17).

In the framework of our experimental *G. lamblia* GS/M-83-H7 infections with the IL-6-deficient mouse model, we explored intestinal anti-*Giardia* IgA production in relation to the course of parasite infection. During the study, 10- to 12-week-old female animals were kept under specific pathogen-free conditions according to Swiss regulations governing animal experimentation and rules for animal protection that restrict the number of animals used experimentally. Experimental infections were done with $10^6$ trophozoites (suspended in 200 μl of a 0.3 M $NaHCO_3$ solution) of *G. lamblia* clone GS/M-83-H7 by using a blunt-ended needle for peroral inoculation (22). The course of *G. lamblia* infection within offspring was determined according to the method of Gottstein et al. (7) by quantitating the parasite burden through microscopic examination of adherent trophozoites from intestinal washes. Antigenic diversification within the intestinal parasite population of infected mice was tested as described earlier by performing an immunofluorescence assay to monitor the switch from MAb G10/4 (VSP H7) positivity to the respective negativity within the affected trophozoite population (9).

For determination of the local anti-*Giardia* IgA concentration, we applied the procedure of Gottstein et al. (7) which is suitable for the extraction of IgA from the intestinal epithelium and lamina propria. The immunoreactivity of intestinal IgA antibodies was tested as described previously (20) by using a total protein extract from *G. lamblia* clone GS/M-83-H7 and/or purified recombinant VSP H7 (the MBP/VSP H7 fusion protein expressed in *Escherichia coli*) as antigenic reagents for enzyme-linked immunosorbent assays (ELISAs) (Fig. 1). In the ELISAs, the maximal variability within triplicate determinations was less than 26%. Immunoblot-based testing of the

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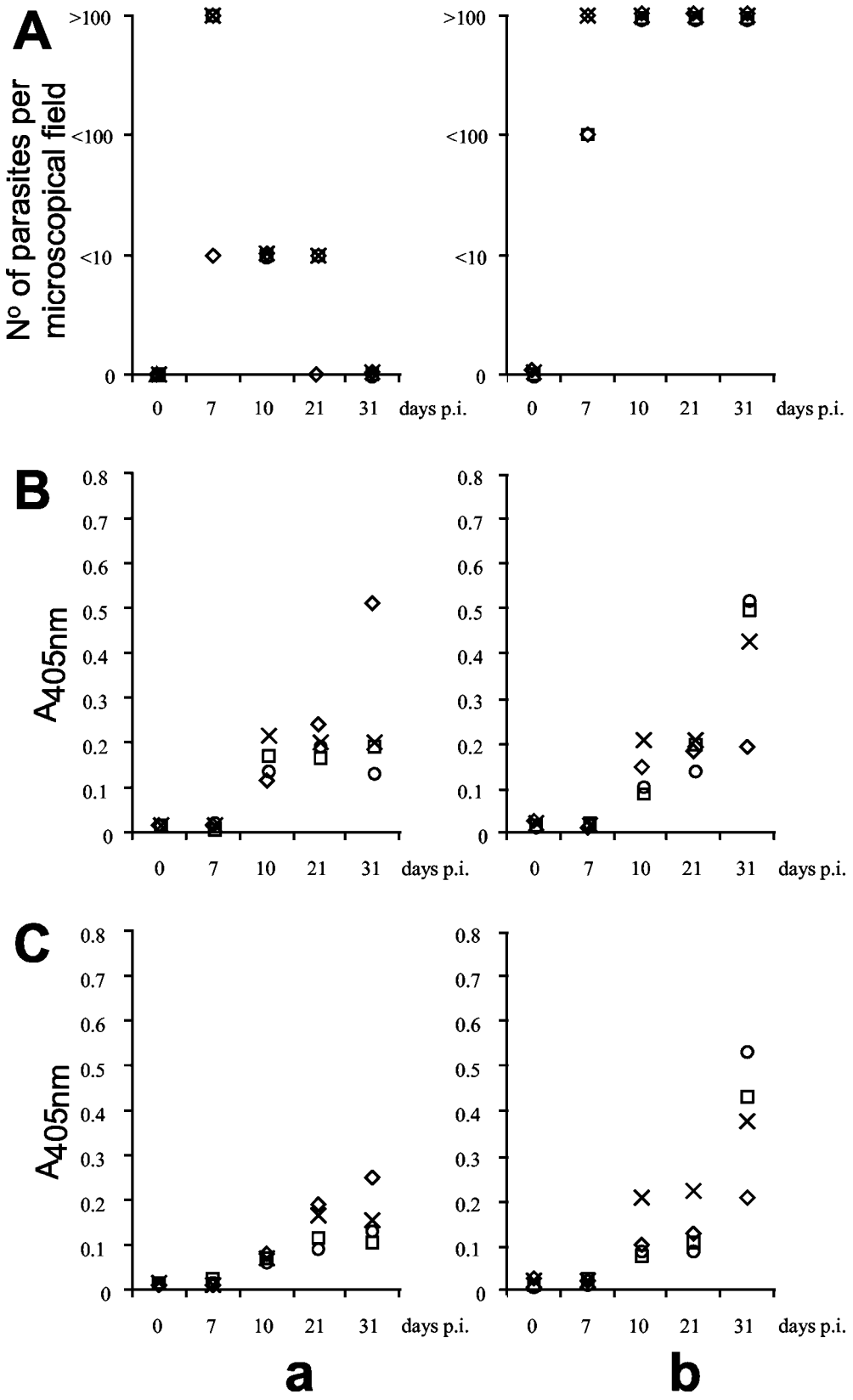


FIG. 1. Follow-up analysis of *G. lamblia* GS/M-83-H7-infected immunocompetent wild-type (a) and IL-6-deficient transgenic (b) mice in terms of detectability of intestinal parasites, monitored by microscopic examination (A) or by ELISA determination of the production of intestinal IgA against purified recombinant VSP H7 protein (B) or total *Giardia* protein (C) and shown as mean values from triplicate determinations. The intestinal parasite burden was semiquantitatively classified as a heavy parasite burden visible as hundreds of parasites (>100) spread over a

intestinal IgA reactivity to total *G. lamblia* GS/M-83-H7 protein was done according to the method of Stäger and Müller (22) (Fig. 2). Time points for sample collection that were suitable for monitoring both the ex vivo antigenic variation of the parasite and the regression of the infection were selected according to the results of a pilot experimental infection of C57BL/6 mice with the parasite (data not shown).

Lymph nodes from four animals per experimental group were pooled and dissociated into single-cell suspensions as previously described (8). Approximately $10^7$ cells were lysed in TRIzol reagent (Invitrogen, Basel, Switzerland), and total cellular RNA was extracted according to the instructions of the manufacturer. Subsequently, residual genomic DNA was removed from 2 μg of RNA by performing a 1-h incubation step at 37°C in the presence of 1 U of RQ1 RNase-free DNase I (Roche Diagnostics, Rotkreuz, Switzerland) and 1× Moloney murine leukemia virus reverse transcription (RT) buffer (Promega). After heat inactivation (5 min at 95°C) of the DNase, RNA preparations were subjected to cDNA synthesis by applying a random oligonucleotide-hexamer primer/Moloney murine leukemia virus reverse transcriptase (Promega) assay according to the instructions of the manufacturer. Finally, cDNA was purified by using a spin column from the High Pure PCR product purification kit (Roche Diagnostics) as recommended by the manufacturer. cDNA was released from the columns by elution with 50 μl of TE (10 mM Tris, 1 mM EDTA), pH 7.6.

Quantitative RT-PCR was carried out on a LightCycler instrument (Roche Diagnostics) by using SYBR Green I as a double-stranded-DNA-specific fluorescent dye and continuous fluorescence monitoring as previously described (23). Amplifications of murine gene sequences from β-actin, cytokines (IL-4, gamma interferon [IFN-γ], IL-2, IL-5, IL-6, and IL-10), and inducible nitric oxide synthase (iNOS) were performed by using primer pairs designed by Overbergh et al. (16). Quantitative PCR was done with 4 μl of 1:4-diluted sample cDNA (see above) by using the LightCycler-FastStart DNA Master SYBR Green I kit in a 10-μl standard reaction supplemented with $MgCl_2$ to a final concentration of 3 mM and containing a 0.5 μM concentration of forward and reverse primers (Invitrogen). All PCRs containing cDNA were performed in triplicate. Furthermore, a control PCR included RNA equivalents from samples that had not been reverse transcribed into cDNA (data not shown) to confirm that no DNA was amplified from any residual genomic DNA that might have resisted DNase I digestion (see above). PCR was started by initiating the HotStart *Taq* DNA polymerase reaction at 95°C (5 min). Subsequent DNA amplification was done in 50 cycles (denaturation [95°C, 0 s], annealing [60°C, 5 s], and extension [72°C, 10 s]; temperature transition rates in all cycle steps were 20°C/s). Fluorescence was measured at the end of each annealing phase in the single mode (channel setting F1), and amplification products were quantitatively assessed by applying the standard

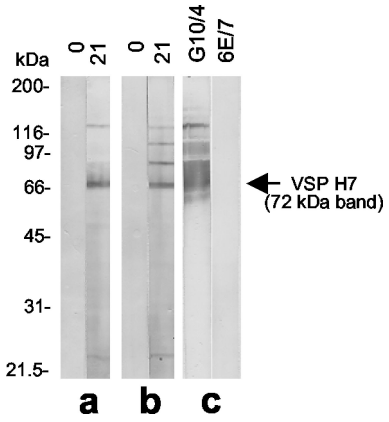


FIG. 2. Immunoblot analysis of parasite-specific intestinal IgA production in *G. lamblia* GS/M-83-H7-infected immunocompetent wild-type mice (lane a) and IL-6-deficient transgenic (lane b) mice. Sodium dodecyl sulfate–10% polyacrylamide electrophoresis gel-fractionated and -blotted total protein from *G. lamblia* GS/M-83-H7 was incubated with intestinal IgA preparations sampled from mice at days 0 and 21 (indicated above lanes a and b as days p.i.) during infection. Reference blots incubated with VSP H7-reactive MAb G10/4 and negative-control MAb 6E7 are also shown (lane c). On the extreme left, sizes of the protein markers are given in kilodaltons. The arrow on the right indicates the major 72-kDa band of variant surface protein H7 (VSP H7).

software (version 3.5.3) of the LightCycler instrument. As external standards, serial 10-fold dilutions (4-μl aliquots) of amplification products previously generated from the different target sequences were included in the quantitative PCR analyses. The standard curves from the different assays (cytokine, iNOS, and β-actin PCRs) were run in duplicate and contained 4 log units within a linear range that essentially covered the maximal and minimal concentrations of the cytokine, iNOS, and β-actin cDNA sequences within the different samples. Linearity among the standard reactions was reflected by the correlation coefficient, which was calculated by the computer program to be extremely high (between 0.99 and 1.0) for all of the PCR assays applied. Furthermore, the efficiencies of the cytokine-, iNOS-, and β-actin-specific quantitative PCRs were revealed to be near identical and exhibited high amplification rates ranging between 1.86 (β-actin PCR) and 1.92 (IL-4 PCR) per cycle. The interassay coefficient of variation did not exceed 15.4%, as it was determined to be for the highest standard dilution of the IL-4 PCR. Overall specificity of the reactions and lack of unwanted primer-dimer formation were confirmed by a DNA melting profile assay (18) using LightCycler standard software (version 3.5.3) and subsequent agarose gel electrophoresis (3% gels), which monitored the PCR products as single DNA bands of expected sizes (data not shown). In order to compensate for the variations in input RNA amounts and efficiencies of RT, the expression of housekeeping gene β-actin was quantitated. Respective mean values from triplicate deter-

microscopic field, a medium parasite burden visible as relatively few parasites (<100) spread over a microscopic field, a low parasite burden visible only as single parasites (<10) by the inspection of one or several microscopic field(s), or no parasites (0), detected by intense microscopic inspection of intestinal washes. Four mice from each group were sacrificed on the indicated number of days p.i. As determined by Student's *t* test for the ELISAs, none of wild-type animal groups exhibited significantly higher antibody concentrations than the corresponding IL-6-deficient animal groups ($P > 0.05$).

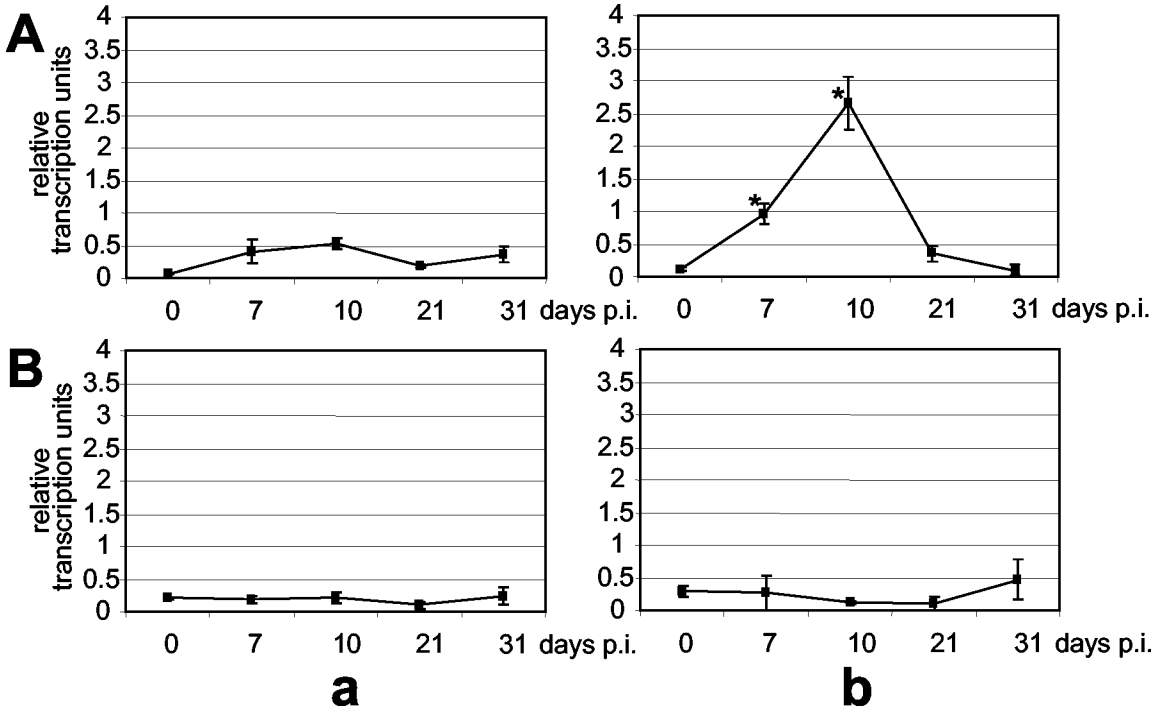


FIG. 3. IL-4 (A) and IFN-γ (B) mRNA levels in peripheral lymph node cells isolated from immunocompetent wild-type (a) and IL-6-deficient transgenic (b) mice at the indicated days during *G. lamblia* GS/M-83-H7 infection were determined by real-time quantitative RT-PCR. The results are given as relative transcription units (cytokine mRNA level/β-actin mRNA level), and standard deviations for triplicate determinations are indicated. Significant differences between the values of the wild-type and corresponding IL-6-deficient animal groups were determined by using Student's *t* test (*, $P < 0.05$).

minations were taken for the calculation of the relative transcription units (cytokine mRNA level/β-actin mRNA level). In both the quantitative RT-PCR assays and ELISAs, the significance of the differences between the wild-type and corresponding IL-6-deficient animal groups was determined by Student's *t* test using the Microsoft Excel program. *P* values of $<0.05$ were considered statistically significant.

Figure 1A shows the results in which the course of the *G. lamblia* infection within the two mouse strains with regard to the infection intensity was monitored. The parasite burden was quantitated by using a previously described method based on the microscopic examination of adherent trophozoites from intestinal washes (7). In early infective stages (around day 7 postinfection [p.i.]), the intestinal parasite burden was relatively high in both wild-type and IL-6-deficient mice. However, at later stages (after day 7 p.i.), infection intensities dramatically diverged between the two experimental animal groups. While the parasite burden dropped to microscopic nondetectability after day 21 p.i. in wild-type animals, the parasite burden remained essentially at the high initial level (>100 trophozoites per microscopical field) in IL-6-deficient animals even at the final experimental time point of infection.

In both wild-type and IL-6-deficient mice, complete antigen switching of trophozoites from MAb G10/4 (VSP H7) positivity to negativity occurred between days 7 and 10 p.i. (data not shown). The ELISA results presented in Fig. 1 reveal that in both mouse strains, production of intestinal anti-VSP H7 IgA (Fig. 1B) and anti-*Giardia* IgA (Fig. 1C) was initiated at approximately the same time point of infection. These two findings confirmed previous data indicating that anti-VSP H7 antibodies have a growth-selective function that promotes antigenic diversification of the parasite in vivo (21, 22). As further assessed in the ELISA shown in Fig. 1, both mouse strains exhibited similar profiles of intestinal anti-VSP H7 and anti-*Giardia* IgA production throughout the entire course of infection investigated. As can be seen in the immunoblot in Fig. 2, the IgA produced by wild-type animals was nearly exclusively directed against VSP H7. IgA produced by IL-6-deficient mice also predominantly recognized the major 72-kDa band of VSP H7. However, particularly in the case of the intestinal IgA preparations sampled at day 21, minor immunoreactivity to additional bands in the higher (positioned in a molecular mass range between about 85 and 120 kDa) molecular mass range was observed. These IgA-reactive, high-molecular-mass proteins most likely represented different complexes and/or folding stages of the VSP H7 peptide (13).

Figure 3 shows the results from the quantitative RT-PCR monitoring of mRNA levels from the Th2 cytokine IL-4 and the Th1 cytokine IFN-γ assessed upon peripheral lymph node cells obtained from *G. lamblia* GS/M-83-H7-infected wild-type and IL-6-deficient mice. The outcome was that IL-4 mRNA levels did not significantly change during infection in wild-type animals (Fig. 3A, panel a). However, IL-6-deficient mice exhibited a massive increase in the respective mRNA levels until day 10 p.i., followed by a rapid decrease down to baseline levels until day 31 p.i. (Fig. 3A, panel b). Conversely, IFN-γ mRNA remained at a constant low level in both animal groups throughout the entire infection period (Fig. 3B). IL-2, IL-5,

IL-6, IL-10, and iNOS mRNAs remained below the level of detectability in all animal groups (data not shown), although the corresponding RT-PCR assays proved their high methodical efficiency in that they demonstrated amplification performances in the standard reactions comparable to those in the IL-4 and IFN-γ RT-PCRs (see also above).

Our experimentation revealed that intestinal IgA production did, from the quantitative and temporal point of view, not significantly differ between wild-type and IL-6-deficient mice. Accordingly, the anti-*Giardia* IgA response occurred in an IL-6-independent manner. This observation is in concordance with a study on *Helicobacter felis* infections in IL-6-deficient mice that also failed to find a critical requirement for IL-6 in mucosal IgA production (5). It is generally accepted that IgA responses require $CD4^+$ Th2 cells producing cytokines that control IgA B-cell differentiation. Among these, IL-4, acting as a major differentiation factor of $CD4^+$ Th2 cells, and IL-5 and IL-6, both involved in the differentiation of committed IgA B cells into IgA-secreting plasma cells, play an important role in mucosal humoral immune responses (3, 17). In our *G. lamblia* infection experiment, an enormous transient elevation of IL-4 expression restricted to IL-6-deficient mice was observed. This overproduction of IL-4 may have promoted Th2-cell differentiation and thus initiated an immunological compensation mechanism that allowed IL-6-deficient mice to develop a normal anti-*Giardia* IgA response. However, this hypothesis needs to be evidenced further in infection experiments investigating the influence of enhanced IL-4 synthesis and the Th2-cell-biased immune reactions on murine anti-*Giardia* IgA production. Th2-cell-biased immune reactions related to murine IL-6 deficiency are well known phenomena that have previously been observed in *Candida albicans* infections (19).

In our study, murine IL-6 deficiency substantially affected the protective immune response against *G. lamblia* GS/M-83-H7 although this defect was not accompanied by reduction of the antibody response towards the parasite. From this observation, we concluded that IL-6 deficiency must have favored antibody-independent mechanisms that prevented the reduction of *G. lamblia* GS/M-83-H7 infection intensity in the murine host. The massive enhancement of the Th2-cell response, as reflected by a strong transient stimulation of IL-4 expression during the initial infection stage, was the only detectable immunological effect associated with susceptibility to infection in IL-6-deficient mice. Accordingly, this transient overexpression of IL-4 may have generated a Th2-cell-dominated immunological environment that enabled persistent growth of the parasite. However, in order to substantiate this hypothesis, additional experimental work will be required that dissects in greater detail the cellular and cytokine profile and the functions of the antigiardial immune response.

We acknowledge A. Hemphill for critical review of the manuscript and T. E. Nash (NIH, Bethesda, Md.) for his gift of the MAbs and *G. lamblia* clone GS/M-83-H7. IL-6-deficient transgenic mice were kindly provided by M. Kopf (Molecular Biomedicine, Department of Environmental Sciences, Swiss Federal Institute of Technology, Zürich, Switzerland).

*Editor:* J. M. Mansfield

This work was supported by grants obtained from the Swiss National Science Foundation (no. 31-58973.99 and 31-066795.01).

M.B. and W.D. contributed equally to this paper.

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